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TITLE OF THE INVENTION

USE OF NOVEL VIRULENCE-SPECIFIC GENES AS TARGETS FOR DIAGNOSIS AND POTENTIAL CONTROL OF VIRULENT STRAINS OF *LISTERIA MONOCYTOGENES*

This application claims priority from U.S. Provisional Application Serial No. 60/444,201, filed February 3, 2003; U.S. Provisional Application Serial No. 60/447,297, filed February 14, 2003; and U.S. Provisional Application Serial No. 60/458,414, filed March 31, 2003. The entirety of each of these provisional applications is incorporated herein by reference.

This invention was made with Government support under 58-0790-0-120 awarded by the U.S. Department of Agriculture-Agricultural Research Service. The Government may have certain rights in the invention.

BACKGROUND OF THE INVENTION

Field of the Invention

This invention involves the use of novel virulence-specific genes of *Listeria monocytogenes* as targets for specific diagnosis and potential control of virulent strains of *L. monocytogenes*. More particularly, this invention provides a PCR or hybridization method, which uses specific primers or probes corresponding to virulence-specific genes for the identification and control of virulent strains of *Listeria monocytogenes*.

Background of the Technology

L. monocytogenes is an important cause of human food borne diseases world wide. A notable feature of *L. monocytogenes* is that it shows considerable variation in its ability to produce listeriosis. On the one extreme, some *L. monocytogenes* strains are virulent and can result in severe disease and mortality. On the other, some have limited capability to establish in the host and are relatively avirulent and harmless. Because manufactured food products detected with *L. monocytogenes* are recalled or downgraded (i.e., used for pet food), contamination with this species may render significant economic losses. With outbreaks of listeriosis due to contaminated foods on the increase in recent years, *L. monocytogenes* has become a major concern to the food industry and health regulation authority.

Apart from adapting stringent quality control measures during food processing procedures, frequent monitoring with specific laboratory tests for virulent strains of *L. monocytogenes* is vital in reducing unnecessary food product recalls and allaying consumer concerns. The current diagnostic methods are incapable of distinguishing virulent from avirulent strains of *L. monocytogenes*.

The complete genome of *Listeria monocytogenes* EGDe strain was reported recently (Glaser et al., 2001). Although this publication contains a list of all known and putative genes in *L. monocytogenes* EGD strain as well as their nucleotide sequences, it does not provide any information on the actual application of these genes. Therefore, although the DNA sequences of the genes described in this invention have been published and are in public domain through the release of the

L. monocytogenes EGDe genome sequence, there are no prior publications on the functions of these genes or on their use for research or diagnostic purposes.

Previous research used PCR and DNA sequencing or restriction fragment length polymorphism of the *L. monocytogenes hlyA*, *actA*, and *inlA* genes to group *L. monocytogenes* into three genetic lineages, with the various lineages varying in potential for human virulence (Norton et al., 2001; Wiedmann et al., 1997). Ribotyping (sequencing of rRNA genes) was also used in this research. These assays are different from the present assay employed by the inventors in that they require either DNA sequencing or restriction digests following PCR amplification, while the present assay is simply a PCR assay. In addition, the *hlyA*, *actA*, and *inlA* genes are present in all *L. monocytogenes* isolates, while the virulence-specific genes described by the inventors are found only in virulent strains of *L. monocytogenes*.

Another PCR assay, random amplification of polymorphic DNA (RAPD) PCR, has been used to classify *L. monocytogenes* into genetic groups that tend to predict virulence. This technique is based on the use of nonspecific primers that bind to unknown sequences in the *L. monocytogenes* chromosome (Franciosa et al., 2001). The PCR assay employed by the inventors is based on primers that bind to specific virulence associated chromosomal sequences that we have identified.

Other assays have been described for differentiation of virulent and avirulent *L. monocytogenes* isolates. The "gold standard" for virulence testing of *L. monocytogenes* isolates is the mouse virulence test. This test is expensive, labor intensive, requires several weeks to complete, and requires regulatory approval to ensure humane treatment of animals. Assays have been described based on cell

culture models; one correlated *L. monocytogenes* virulence with the ability of isolates to form plaques on HT-29 cells (Roche et al., 2001), and another correlated virulence with the ability to cause cytopathogenic effects in Caco-2 cells (Pine et al., 1991). Although the use of cell culture models represents an improvement over mouse virulence testing, it is still time-consuming and labor intensive.

Research has been published on the use of phenotypic detection of virulence factor expression (listeriolysin, phosphatidylinositol phospholipase C, phosphatidylcholine phospholipase C) to separate virulent from avirulent *L. monocytogenes* isolates (Erdenlig et al., 2000). Research has also been published on the use of monoclonal antibodies for detection of virulence factor expression (listeriolysin and phosphatidylcholine phospholipase C) to distinguish virulent and avirulent isolates (Erdenlig et al., 1999). The dot blot hybridization technique described in this invention has also been previously published. For example, this technique was employed to identify virulence and avirulence associated markers of *Dichelobacter nodosus* - the ovine footrot pathogen (Liu & Yong, 1993). Several PCR assays have been described for species specific detection of *L. monocytogenes* (examples include Aznar & Alarcon, 2002; Bassler et al., 1995; Blais et al., 1997; Klein & Juneja, 1997; Norton & Batt, 1999; Winters et al., 1999). PCR assays for distinguishing all six *Listeria* species can be based on the 16S and 23S rRNA genes (Sallen et al., 1996) or the intergenic spacer region of 16S and 23S rRNA genes (Graham et al., 1997), or the *iap* gene (Bubert, et al., 1999). However, none of these PCR assays distinguish virulent *L. monocytogenes* isolates from avirulent isolates.

SUMMARY OF THE INVENTION

This invention involves the use of virulence-specific genes of *Listeria monocytogenes* as targets for specific diagnosis and potential control of virulent strains of *L. monocytogenes* and overcomes the above identified shortcomings of conventional detection methods.

Using a comparative screening strategy, the inventors isolated two potential virulence-specific clones from a recombinant DNA library from *L. monocytogenes* strain EGD. Specifically, a hybridization technique was used to compare genomic DNA from virulent and avirulent *L. monocytogenes* isolates to identify clones containing genetic markers that are uniquely present in either virulent and/or avirulent strains. DNA sequence analysis of the two virulence specific clones revealed that they contain gene markers that are distinct from the previously reported virulence gene cluster encompassing *prfA*, *plcA*, *hlyA*, *mpl*, *actA, and plcB*. By employing primers derived from these as well as other newly identified virulence-specific gene markers, the inventors discovered a method by which virulent strains of *L. monocytogenes* can now be readily distinguished from avirulent strains through the formation of specific PCR products.

The method of the present invention for separation of virulent and avirulent *L. monocytogenes* isolates can be used to provide a scientific basis for the determination of when and if food safety recalls should occur when *L. monocytogenes* is isolated from food products.

In one embodiment of this invention, virulence-specific genes of *Listeria monocytogenes* are used as targets for specific diagnosis and potential control of virulent strains of *L. monocytogenes*.

In another embodiment of this invention, one or more of *L. monocytogenes* virulence-specific genes are used to detect virulent strains of *L. monocytogenes*.

In another embodiment of this invention, one or more of *L. monocytogenes* virulence-specific genes are used to detect virulent strains of *L. monocytogenes* by polymerase chain reaction (PCR) using primers specific for the DNA sequence from the gene(s) or by hybridization using a probe specific for the DNA sequence from the gene(s).

In another embodiment of this invention, the one or more *L. monocytogenes* virulence-specific genes are selected from the group consisting of: *lmo0833*, *lmo2672*, *lmo1116*, and *lmo1134* (encoding putative transcriptional regulators); *lmo0834* and *lmo1188* (encoding proteins with unknown function); and *lmo0333*, *lmo2470*, and *lmo2821* (encoding proteins similar to internalins).

In another embodiment of this invention, a combination of two or more of *L. monocytogenes* virulence-specific genes are used to detect virulent strains of *L. monocytogenes* by multiplex polymerase chain reaction (PCR) or hybridization using primers or probes specific for the DNA sequences from the gene(s).

In another embodiment of this invention, one or more of *L. monocytogenes* virulence-specific genes are used to detect virulent strains of *L. monocytogenes* by multiplex polymerase chain reaction (PCR) or hybridization using primers or probes specific for the DNA sequence from the gene(s) in combination with *Listeria* genus-specific primers or probes and/or *L. monocytogenes* species-specific primers or probes.

In another embodiment of this invention, the one or more *L. monocytogenes* virulence-specific genes are one or more genes that indicate virulent forms of *L. monocytogenes* or combinations thereof.

In another embodiment of this invention, the *L. monocytogenes* virulence-specific genes or their derivatives are used in the inhibition of growth, reduction of pathogenicity, treatment, or prevention of virulent strains of *Listeria monocytogenes*.

In another embodiment of this invention, virulent strains of *Listeria monocytogenes* are detected by amplification of *L. monocytogenes* virulence-specific genes from mRNA by reverse transcription-PCR (RT-PCR).

In another embodiment of this invention, virulent strains of *Listeria monocytogenes* are detected by one or more methods for detection of protein product(s) from *L. monocytogenes* virulence-specific genes.

In another embodiment of this invention, virulent strains of *Listeria monocytogenes* are detected by one or more methods for detection of protein product(s) from *L. monocytogenes* virulence-specific genes using either polyacrylamide gel electrophoresis, high-performance liquid chromatography (HPLC), mass spectrometry, or antibody detection methods (examples include immunofluorescent antibodies (IFA), enzyme-linked immunosorbent assay (ELISA), or Western blotting).

In another embodiment of this invention, virulent strains of *Listeria monocytogenes* are detected by one or more methods for detection of protein product(s) from *L. monocytogenes* virulence-specific genes by use of assay(s) specific for the function(s) of the protein product(s).

In another embodiment of this invention, the virulence-specific *L. monocytogenes* genes are used as a treatment strategy such that pharmaceutically active agent(s) would inactivate or alter the function of one or more of the proteins encoded by the virulence-specific *L. monocytogenes* genes, which would either kill the virulent *L. monocytogenes* or render it susceptible to the host immune system.

In another embodiment of this invention, one or more of the *L. monocytogenes* genes or promoter(s) for one or more of the virulence-specific *L. monocytogenes* genes is altered such that expression of the encoded protein(s) would be completely disrupted or altered. The said alteration or disruption of expression would render *L. monocytogenes* avirulent and effective as a live attenuated vaccine.

In another embodiment of this invention, the *L. monocytogenes* virulence-specific genes are selected from the group consisting of: *lmo0833*, *lmo1188*, *lmo0834*, *lmo1116*, *lmo2672*, *lmo1134*, *lmo0333*, *lmo2470*, and *lmo2821*.

In another embodiment of this invention, the one or more *L. monocytogenes* virulence-specific genes are one or more genes that indicate one or more virulent forms of *L. monocytogenes*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the DNA sequences of SEQ ID NOS.: 1-9 for each of the virulence-specific genes of *Listeria monocytogenes* according to the present invention.

Figure 2 shows the DNA sequences of SEQ ID NOS.: 28-33 for each of the *Listeria* species-specific gene sequences according to the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Listeria monocytogenes is a small gram-positive coccobacillus that tends to form short chains of three to five bacteria. Infections from this pathogen occur worldwide in various animals and man (Gray and Killinger, 1966) and can be fatal in immunocompromised individuals such the elderly, pregnant women, newborns, diabetics and transplantation patients (Gellin and Broome, 1989). *L. monocytogenes* is of particular concern to the food industry and public health regulatory agencies because it can grow at refrigerator temperatures and because it is ubiquitous in nature (Farber and Speirs, 1987, Lamont et al., 1988). It has been found in a variety of foods such as vegetables (Heisick et al., 1989), milk (Donnelly and Baigent, 1986, Doyle et al., 1987), various cheeses (Rodler and Korbler, 1989), meat products (Farber et al., 1989), poultry (Carpenter and Harrison, 1989), and fish (Lennon et al., 1984; Erdenlig et al., 1999).

Of the 13 known serotypes of *L. monocytogenes*, many of which are found in foods, only three serotypes (1/2a, 1/2b, 4b) are associated with the majority of human illness (Schuchat et al., 1991). However, not all strains of these *L. monocytogenes* serotypes are pathogenic, with some strains having either no or low-level virulence (Hof and Rocourt, 1992). Previous work at the College of Veterinary Medicine at Mississippi State University indicated that *L. monocytogenes* isolates from channel catfish vary in virulence using the mouse model, with some isolates being highly virulent and others being completely

avirulent (Erdenlig et al., 2000). There is also molecular evidence for the existence of genetic lineages of *L. monocytogenes* that vary in virulence (Norton et al., 2001; Wiedmann et al., 1997). This data indicates that food safety recalls based solely on detection of *L. monocytogenes* without determination of virulence could lead to unnecessary recalls, which would have devastating consequences on food producers and processors. To prevent economic losses due to food recalls, and to reduce human food safety concerns, it is important to understand what causes certain *L. monocytogenes* to be virulent and to devise ways to accurately ascertain virulence.

L. monocytogenes is a facultative intracellular pathogen, and some of its best-known virulence factors contribute to its ability to survive inside professional phagocytic cells. After it is phagocytosed, *L. monocytogenes* lyses the host vacuole and escapes into the cell cytoplasm. This step is mediated by listeriolysin (LLO) and phosphatidylinositol phospholipase C (PI-PLC) (Camilli et al., 1993, Portnoy et al., 1988). The bacteria are then propelled through the host cell cytoplasm by inducing the polymerization of host actin, a process that is mediated by a surface protein designated ActA (Domann et al., 1992). The bacteria then apparently spread from cell to cell by inducing formation of pseudopod-like structures containing bacteria that are internalized by neighboring cells. A second phospholipase, phosphatidylcholine phospholipase C (PC-PLC) is required for this step (Vazquez-Boland et al., 1992). A zinc metalloprotease, Mpl, may be required for activation of PC-PLC (Poyart et al., 1993).

The genes encoding these virulence factors are clustered on the *L. monocytogenes* chromosome between the *ldh* and *prs* operons: *prfA* (PrfA,

regulatory gene), *plcA* (PI-PLC), *hlyA* (LLO), *mpl* (Mpl), *actA* (ActA), and *plcB* (PC-PLC) (Portnoy et al., 1992). This gene cluster is one of the most well studied regions of the *L. monocytogenes* chromosome; there have been numerous publications on the roles that these genes play in virulence (Bohne et al., 1996, Bubert et al., 1999, Freitag and Jacobs, 1999, Kuhn and Goebel, 1995, Smith et al., 1995).

Previous work at the College of Veterinary Medicine at Mississippi State University has shown that expression of LLO and PC-PLC is valuable in indicating the pathogenicity of *L. monocytogenes* isolates (Erdenlig et al., 1999; Erdenlig et al., 2000). Expression of LLO and PC-PLC in seven *L. monocytogenes* isolates were compared, four of which were virulent in mice and three of which were avirulent in mice. Expression of both LLO and PC-PLC was present in all four virulent strains, and expression of LLO and PC-PLC was absent in two out of three avirulent strains (Table 1). None of the three avirulent strains expressed both LLO and PC-PLC.

Table 1. Application of mAbs to detect the presence of virulence factors from *L. monocytogenes* channel catfish isolates and their correlation to pathogenicity

L. monocytogenes catfish isolate	Serovar	LLO	PC-PLC	Pathogenicity[1]
ATCC 15313	1	-	+	-
ATCC 19115	4b	+	+	+
EGD	1/2a	+	+	+
CCF 1[2]	1	+	+	+
HCC 7[2]	1	+[3]	+	+
HCC23	4	+	-	-

[1] Pathogenicity data for CCF 1, CCF 4, HCC 7, and HCC 23 are published in Erdenlig et al. (1999).

[2] CCF = channel catfish fillet; HCC = healthy channel catfish organs.

[3] HCC 7 is weakly positive for LLO.

DNA sequencing of the promoters from the virulence gene clusters of these seven *L. monocytogenes* isolates were completed. The promoters that were sequenced control expression of the *hlyA*, *plcA*, *prfA*, and *plcB* genes. In addition, the entire *prfA* gene was sequenced from the seven isolates because PrfA binds to each of these promoters to control transcription. The sequences were obtained by first amplifying the regions of interest by PCR and directly sequencing the PCR products.

The sequencing results provide evidence that there are distinct genetic lineages of *L. monocytogenes* based on the virulence gene promoter sequences. Phylogenetic analysis indicated that in three of the promoters, the seven strains grouped consistently into three genetic lineages. In the fourth promoter controlling hly (LLO) expression, five out of seven isolates were grouped into the same genetic lineages. The different groupings of the other two strains at this promoter possibly reflect differences in expression of LLO.

The sequencing results also revealed potential sequence differences that could explain the differential expression of LLO and PC-PLC between isolates. In one isolate that fails to express PC-PLC, two amino acid substitutions were detected in PrfA. In the hly promoter, there were three nucleotide substitutions in the strain that fails to produce LLO compared to other strains. In one of the *plcB* promoters, there were four nucleotide substitutions in the promoter region of a non-PC-PLC producing strain compared to other strains.

However, the sequencing results also demonstrated that these genes (*prfA*, *plcA*, *hlyA*, *and plcB*) are not good candidates for the development of PCR-based

tests for distinguishing virulent from avirulent strains. These genes are present in all *L. monocytogenes* isolates (and even some other *Listeria* species), and the sequencing results demonstrated that the sequence variations in these genes between virulent and avirulent isolates are too few to allow development of PCR primers that would reliably distinguish virulent and avirulent isolates.

Therefore, the goal was to identify other gene markers that could be used for distinguishing virulent *L. monocytogenes* isolates from avirulent isolates. Although the genome sequence of virulent *L. monocytogenes* strain EGD recently became available (Glaser et al., 2001), the sequence of avirulent *L. monocytogenes* isolates are not available for comparison to identify these unique genes. Therefore, dot blot hybridization was used to identify *L. monocytogenes* virulence-associated markers, which is a technique that had been previously used to detect chromosomal markers that are unique to both virulent and avirulent isolates of *Dichelobacter nodosus*, the causative agent of ovine footrot (Liu and Yong, 1993). These markers identified from *D. nodosus* were used as the basis for development of a diagnostic test that can be used to differentiate virulent, intermediate, and avirulent isolates of this species (Liu, 1994).

To prepare for dot blot hybridization, genomic DNA was prepared from the known virulent and avirulent strains of *Listeria monocytogenes* using a standard protocol (Ausubel et al., 1994) and suspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The purified DNA from virulent strain EGD and avirulent strain HCC23 was partially digested with restriction endonuclease Sau3A I. Digested DNA was separated by agarose gel electrophoresis, and fragments in the 0.5-3 kilobase range were excised and eluted. The size fractionated DNA was then

cloned into BamH I digested plasmid vector (pGEM-3Z; Promega). The resultant recombinant DNA libraries were transformed into *E. coli* XL1-Blue MRF, and clones with insert were identified by blue-white screening. Plasmid DNA was isolated from individual clones in batches of 50 using a rapid alkaline lysis procedure. Inserts were isolated by digestion with Pst I and EcoR I, separated from vector DNA by agarose gel electrophoresis, eluted by the phenol-thaw method, and labeled for hybridization using the ECL protocol for labeling double stranded DNA (Amersham Pharmacia Biotech). If inserts contained Pst I or EcoR I restriction sites, inserts were recovered by digestion with Sma I and Hind III.

The dot blot hybridization was conducted using the procedure described by Liu and Yong (Liu and Yong, 1993). Briefly, DNA from each of the four virulent strains and the three avirulent strains were heated at 100 °C for 3 minutes before being mixed with an equal volume of 1.8 M NaCl, 0.18 M sodium citrate and 4.4 M formaldehyde. Fifty microliters of DNA from each of the seven strains (0.5 g DNA/dot) was spotted onto nylon membranes (Hybond-N, Amersham Pharmacia Biotech) using a dot blot apparatus (Schleicher and Schuell). DNA was spotted in 50 panels, with each panel containing one dot from each of the seven strains, and fixed on membranes using UV light in a Stratalinker 2400 (Stratagene). Dot blot panels were separated from each other and individually hybridized with the labeled inserts.

Inserts were identified from these hybridizations that demonstrate preferential binding to virulent or avirulent strains. Inserts from identified clones were sequenced on both ends using primers from the vector sequence. Clones from the virulent strain EGD were easily identified based on the available genome

sequence data, but inserts from the avirulent strain required sequencing the entire insert using a primer walking strategy. Southern hybridizations using labeled probes from the identified clones were conducted using genomic DNA from all seven strains to confirm results from the dot blot hybridizations.

Through this comparative screening procedure, two recombinant clones (Lmo2-28 and Lmo2-432) were identified from the genomic DNA libraries of *L. monocytogenes* strain EGD (NCTC7973). Following nucleotide sequence analysis of these two clones and subsequent BLAST searches at GenBank, clone Lmo2-28 was found to contain parts of *lmo0833*/*lmo0834* of *L. monocytogenes* EGDe, which encode a putative transcriptional regulator and an unknown protein. Clone Lmo2-432 was found to contain part of lmo1188 of *L. monocytogenes* EGDe, which encodes an unknown protein. Because of this interesting finding and the fact that transcriptional regulators are specialized DNA binding proteins that play essential roles in the regulation of RNA synthesis and gene expression within bacteria, attention was focused on genes encoding transcriptional regulators in *L. monocytogenes*. As a result, several other genes (*lmo2672*, *lmo1116*, and *lmo1134*) were selected from the list of *L. monocytogenes* EGDe genes (Glaser et al., 2001) for further evaluation (Table 2). Furthermore, because internalins are found exclusively in *Listeria*, additional attention was also directed to *L. monocytogenes* EGDe genes that encode putative internalins. Thus, the inventors selected three genes (*lmo0333*, *lmo2470*, and *lmo2821*) that code for proteins similar to internalins for assessment. The *Listeria monocytogenes* virulence-specific genes used as examples of the present invention are listed in Table 2. Sequence lists for each of these genes are shown in Figure 1 as: *lmo0833* (SEQ ID NO: 1), *lmo1188*

(SEQ ID NO: 2), *lmo0834* (SEQ ID NO: 3), *lmo1116* (SEQ ID NO: 4), *lmo2672* (SEQ ID NO: 5), *lmo1134* (SEQ ID NO: 6), *lmo0333* (SEQ ID NO: 7), *lmo2470* (SEQ ID NO: 8), and *lmo2821* (SEQ ID NO: 9). Primers [forward primers (5'-3') and reverse primer (3'-5')], corresponding to each of the *L. monocytogenes* virulence-specific genes are also shown in Table 2. As indicated in the Table, these primers are sequentially designated as SEQ ID NOS.: 10-27. The oligonucleotide primers, which were designed from each of these genes were assessed in PCR against a collection of 29 *L. monocytogenes* strains (Table 3).

Table 2. Identities of novel *L. monocytogenes* virulence specific gene markers

Gene	Genome location	Putative function	Size (aa)	Forward primer (5'-3')	Reverse primer (5'-3')	Primer positions	PCR product (bp)
lmo0833	223780-224730	Transcriptional regulator	296	ggctattctttagcggagga SEQ ID NO. 10	agtagcgcgagggatttgta SEQ ID NO. 11	223996-224015; 224633-224613	638
lmo1188	53621-55085	Unknown protein	483	tttcgccgttagaaaatacga SEQ ID NO. 12	ttcggacaaaaatttgaatgg SEQ ID NO. 13	54027-54047; 54689-54668	663
lmo0834	224810-225537	Unknown protein	237	aacttcgcatttgttatgtgttac SEQ ID NO. 14	tcactgaccattcctccaaa SEQ ID NO. 15	224940-224963; 225533-225513	594
lmo1116	262997-263783	Transcriptional regulator	257	gggaacgatgaaaacgaaga SEQ ID NO. 16	tggcttatcgcacaagctaat SEQ ID NO. 17	263006-63025; 263593-263573	591
lmo2672	25985-26804	Transcription regulator	268	cggcacacttggattctcat SEQ ID NO. 18	agggctagtgacggatgcta SEQ ID NO. 19	26117-26136; 26597-26578	481
lmo1134	8009-8368	Transcriptional regulator	115	acccgatagcaaggaggaac SEQ ID NO. 20	aacttctctcgatacccatcca SEQ ID NO. 21	7998-8017; 8364-8343	367
lmo0333	936-6272	Internalin	1778	ccgatttagaaacgcttgga SEQ ID NO. 22	ttcggcatatcgtgaatcat SEQ ID NO. 23	1930-1949; 2569-2550	640
lmo2470	149254-150433	Internalin	388	tgattccatgcaattactagaacg SEQ ID NO. 24	aggattctaaactaggtaagttggtg SEQ ID NO. 25	149527-149550; 150071-150046	545
lmo2821	188153-190708	Internalin	851	tgtaaccccgcttacacagtt SEQ ID NO. 26	ttacggctggattgtctgtg SEQ ID NO. 27	188989-189009; 189599-189580	611

Table 3. List of bacterial strains examined by PCR using *L. monocytogenes* virulence specific primers

Strain	Serovar	*lmo0833/ lmo1188*	*lmo0834*	*lmo1116*	*lmo2672/ lmo1134*	*lmo0333*	*lmo2470*	*lmo2821*
L. monocytogenes ATCC 19111	1	+	+	+	+	+	+	+
L. monocytogenes ATCC 19112	2	+	+	+	+	+	+	+
L. monocytogenes ATCC 19113	3	+	+	+	+	+	+	+
L. monocytogenes ATCC 19114	4a	-	-	-	-	-	-	-
L. monocytogenes ATCC 19115	4b	-	+	-	+	+	+	+
L. monocytogenes ATCC 19116	4c	-	-	+	-	-	-	+
L. monocytogenes ATCC 19117	4d	-	+	+	+	-	+	+
L. monocytogenes ATCC 19118	4e	-	-	+	+	-	+	+
L. monocytogenes ATCC 15313	1	+	-	+	+	-	+	+
L. monocytogenes EGD (NCTC 7973)	1/2a	+	+	+	+	+	+	+
L. monocytogenes HCC7	1	+	+	+	+	+	+	+
L. monocytogenes HCC8	1	+	+	+	+	-	+	+
L. monocytogenes HCC12	4	-	-	-	-	-	-	-
L. monocytogenes HCC13	4	-	-	-	-	-	-	-
L. monocytogenes HCC16	4	-	-	-	-	-	-	-
L. monocytogenes HCC17	4	-	-	-	-	-	-	-
L. monocytogenes HCC18	4	-	-	-	-	-	-	-
L. monocytogenes HCC19	4	-	-	-	-	-	-	-
L. monocytogenes HCC23	4	-	-	-	-	-	-	-
L. monocytogenes HCC24	4	-	-	-	-	-	-	-
L. monocytogenes HCC25	4	-	-	-	-	-	-	-
L. monocytogenes 168		+	+	+	+	+	+	+
L. monocytogenes 180		+/-	+	-	+	+	+	+
L. monocytogenes 418		+	+	-	+	+	+	+
L. monocytogenes 742		+	+	+	+	+	+	+
L. monocytogenes 874		-	-	-	-	+	+	+
L. monocytogenes 1002		+	+	+	+	+	+	+
L. monocytogenes 1084		+	+	+	+	-	+	+
L. monocytogenes 1400		+	+	+	+	+	+	+
L. innocua ATCC 33090	6a	-	-	-	-	-	-	-
L. innocua 415		-	-	-	-	-	-	-
L. innocua 416		-	-	-	-	-	-	-

Strain	Serovar	*lmo0833/ lmo1188*	*lmo0834*	*lmo1116*	*lmo2672/ lmo1134*	*lmo0333*	*lmo2470*	*lmo2821*
L. innocua 417		-	-	-	-	-	-	-
L. innocua 662		-	-	-	-	-	-	-
L. innocua 1419		-	-	-	-	-	-	-
L. innocua 1425		-	-	-	-	-	-	-
L. innocua 1720		-	-	-	-	-	-	-
L. innocua 1944		-	-	-	-	-	-	-
L. grayi ATCC 19120		-	-	-	-	-	-	-
L. grayi ATCC 25400		-	-	-	-	-	-	-
L. murrayi ATCC 25401		-	-	-	-	-	-	-
L. ivanovii ATCC 19119		-	-	-	-	-	-	-
L. ivanovii 3325		-	-	-	-	-	-	-
L. seeligeri ATCC 35967		-	-	-	-	-	-	-
L. seeligeri 3008		-	-	-	-	-	-	-
L. seeligeri 3321		-	-	-	-	-	-	-
L. welshimeri ATCC 35897		-	-	-	-	-	-	-
L. welshimeri ATCC 43550	1/2b	-	-	-	-	-	-	-
L. welshimeri ATCC 43551	6a	-	-	-	-	-	-	-
L. welshimeri CCF4		-	-	-	-	-	-	-
L. welshimeri 1471		-	-	-	-	-	-	-
Aeromonas hydrophila ATCC 35654		-	-	-	-	-	-	-
Clostridium perfringens		-	-	-	-	-	-	-
Enterococcus faecalis ATCC 29212		-	-	-	-	-	-	-
Escherichia coli ATCC 25922		-	-	-	-	-	-	-
Flavobacterium indolegenes		-	-	-	-	-	-	-
Klebsiella pneumoniae ATCC 13883		-	-	-	-	-	-	-
Proteus vulgaris ATCC 13315		-	-	-	-	-	-	-
Pseudomonas aeruginosa ATCC 27853		-	-	-	-	-	-	-
Salmonella typhimurium ATCC 14028		-	-		-	-	-	-
Serratia marcescens ATCC 8100		-	-	-	-	-	-	-
Staphylococcus aureus ATCC 25923		-	-	-	-	-	-	-
Streptococcus pneumoniae		-	-	-	-	-	-	-
Streptococcus pyogenes ATCC 19615		-	-	-	-	-	-	-
Vibrio cholerae		-	-	-	-	-	-	-
Yersinia pseudotuberculosis		-	-	-	-	-	-	-

The results indicated that the PCR primers derived from these genes reacted predominantly with virulent strains of *L. monocytogenes* because the virulence of several of these strains (EGD, 19115, CCF1, HCC7, HCC23 and 15313) was determined previously by mouse virulence assay (Erdenlig et al., 2000). To further verify the virulence of *L. monocytogenes* strains as determined by PCR, a second mouse virulence trial was recently conducted involving 12 *L. monocytogenes* strains (Table 4). The validity of PCR determination of the virulence of *L. monocytogenes* has been again confirmed by the mouse virulence trial. One notable exception is *L. monocytogenes* strain ATCC15313, which is avirulent due to a mutation that causes failure to express listeriolysin, a known virulence factor. The PCR results suggest that the other virulence-specific genes in this strain are intact.

Table 4. Summary of *L. monocytogenes* mouse virulence trial

Strain	***Serovar***	***PCR***	***Mouse virulence trial***	***LD50***
L. monocytogenes ATCC 19112	2	V	V	$1.6x10^{9}$
L. monocytogenes ATCC 19114	4a	A	A	$1.9x10^{10}$
L. monocytogenes ATCC 19115	4b	V	V	$6.0x10^{8}$
L. monocytogenes ATCC 19116	4c	V	V	$2.6x10^{8}$
L. monocytogenes ATCC 19117	4d	V	V	$8.8x10^{8}$
L. monocytogenes ATCC 19118	4e	V	V	$7.8x10^{9}$
L. monocytogenes ATCC 15313	1	V	A	$>1.2x10^{11}$
L. monocytogenes EGD	1/2a	V	V	$<1.1x10^{7}$
L. monocytogenes HCC8	1	V	V	$<7x10^{8}$
L. monocytogenes HCC25	4	A	A	$3.5x10^{10}$
L. monocytogenes 874	not determined	V	V	$<8.0x10^{7}$
L. monocytogenes 1002	not determined	V	V	$5.2x10^{8}$

Therefore, the present invention utilizes one or more *L. monocytogenes* virulence-specific genes that allow detection of virulent strains of *L.*

monocytogenes. Specifically, these genes include *lmo0833*, *lmo2672*, *lmo1116*, and *lmo1134* (encoding putative transcriptional regulators); *lmo0834*, and *lmo1188* (encoding proteins with unknown function); and *lmo0333*, *lmo2470, and lmo2821* (encoding proteins similar to internalins). Indeed, the combined use of *lmo2470* and *lmo1116*; or *lmo0333* and *lmo1116*, or the use of *lmo2821* alone is sufficient to enable identification of all potentially virulent *L. monocytogenes* strains under investigation. The scope of this invention also includes other genes identified by the methods described that could indicate virulent forms of *L. monocytogenes*. For example, the described techniques have been used to identify other genetic markers unique to *L. monocytogenes* , *L. innocua*, *L. grayi*, *L. ivanovii* , *L. seeligeri* and *L. welshimeri* (Table 5), that could be used for the development of species-specific PCR assays. These species-specific PCR assays have been tested against a panel of *Listeria* and other gram-positive and negative species.

Table 5. List of bacterial strains examined in PCR using *Listeria* species-specific primers

Strain	Serovar	Source	*lmo0733* (455 bp)	*lin0464* (749 bp)	Lgr20-246 (420 bp)	Liv22-228 (467 bp)	Lse24-315 (371 bp)	Lwe7-571 (608 bp)
L. monocytogenes ATCC 19111	1	Poultry	+	-	-	-	-	-
L. monocytogenes ATCC 19112	2	Human	+	-	-	-	-	-
L. monocytogenes ATCC 19113	3	Human	+	-	-	-	-	-
L. monocytogenes ATCC 19114	4a	Human	+	-	-	-	-	-
L. monocytogenes ATCC 19115	4b	Human	+	-	-	-	-	-
L. monocytogenes ATCC 19116	4c	Chicken	+	-	-	-	-	-
L. monocytogenes ATCC 19117	4d	Sheep	+	-	-	-	-	-
L. monocytogenes ATCC 19118	4e	Chicken	+	-	-	-	-	-
L. monocytogenes ATCC 15313	1	Rabbit	+	-	-	-	-	-
L. monocytogenes EGD (NCTC7973)	1/2a	Human	+	-	-	-	-	-
L. monocytogenes HCC7	1	Catfish brain	+	-	-	-	-	-
L. monocytogenes HCC8	1	Catfish brain	+	-	-	-	-	-
L. monocytogenes HCC12	4	Catfish brain	+	-	-	-	-	-
L. monocytogenes HCC13	4	Catfish kidney	+	-	-	-	-	-
L. monocytogenes HCC16	4	Catfish brain	+	-	-	-	-	-
L. monocytogenes HCC17	4	Catfish brain	+	-	-	-	-	-
L. monocytogenes HCC18	4	Catfish spleen	+	-	-	-	-	-
L. monocytogenes HCC19	4	Catfish spleen	+	-	-	-	-	-
L. monocytogenes HCC23	4	Catfish brain	+	-	-	-	-	-
L. monocytogenes HCC24	4	Catfish spleen	+	-	-	-	-	-
L. monocytogenes HCC25	4	Catfish kidney	+	-	-	-	-	-
L. monocytogenes 168		Aborted calf fetus	+	-	-	-	-	-
L. monocytogenes 180		Human outbreak	+	-	-	-	-	-
L. monocytogenes 418		Freezer study	+	-	-	-	-	-
L. monocytogenes 742		Ground beef	+	-	-	-	-	-
L. monocytogenes 874		Cow brain	+	-	-	-	-	-
L. monocytogenes 1002		Pork sausage	+	-	-	-	-	-
L. monocytogenes 1084		Chicken	+	-	-	-	-	-

Strain	Serovar	Source	*lmo0733* (455 bp)	*lin0464* (749 bp)	Lgr20-246 (420 bp)	Liv22-228 (467 bp)	Lse24-315 (371 bp)	Lwe7-571 (608 bp)
L. monocytogenes 1400		Jalisco outbreak	+	-	-	-	-	-
L. innocua ATCC 33090	6a	Cow brain	-	+	-	-	-	-
L. innocua 415		Turkey burger	-	+	-	-	-	-
L. innocua 416		Veal/beef patty	-	+	-	-	-	-
L. innocua 417		Beef steak	-	+	-	-	-	-
L. innocua 662		Raw milk	-	+	-	-	-	-
L. innocua 1419		Ground cheese	-	+	-	-	-	-
L. innocua 1425		Pecorino Romano	-	+	-	-	-	-
L. innocua 1720		Chicken	-	+	-	-	-	-
L. innocua 1944		Ground turkey	-	+	-	-	-	-
L. grayi ATCC 19120		Chinchilla faeces	-	-	+	-	-	-
L. grayi ATCC 25400		Corn leaves/stalks	-	-	+	-	-	-
L. murrayi ATCC 25401		Corn leaves/stalks	-	-	+	-	-	-
L. ivanovii ATCC 19119		Sheep	-	-	-	+	-	-
L. ivanovii 3325		Cheese	-	-	-	+	-	-
L. seeligeri ATCC 35967		Soil	-	-	-	-	+	-
L. seeligeri 3008		Soil	-	-	-	-	+	-
L. seeligeri 3321		Cheese	-	-	-	-	+	-
L. welshimeri ATCC 35897		Plant	-	-	-	-	-	+
L. welshimeri ATCC 43550	1/2b	Soil	-	-	-	-	-	+
L. welshimeri ATCC 43551	6a	Soil	-	-	-	-	-	+
L. welshimeri CCF4		Catfish brain	-	-	-	-	-	+
L. welshimeri 1471		Environment	-	-	-	-	-	+
Aeromonas hydrophila ATCC 35654			-	-	-	-	-	-
Clostridium perfringens		Clinical	-	-	-	-	-	-
Enterococcus faecalis ATCC 29212			-	-	-	-	-	-
Escherichia coli ATCC 25922			-	-	-	-	-	-
Flavobacterium indolegenes		Clinical	-	-	-	-	-	-
Klebsiella pneumoniae ATCC 13883			-	-	-	-	-	-

Strain	Serovar	Source	*lmo0733* (455 bp)	*lin0464* (749 bp)	Lgr20-246 (420 bp)	Liv22-228 (467 bp)	Lse24-315 (371 bp)	Lwe7-571 (608 bp)
Proteus vulgaris ATCC 13315			-	-	-	-	-	-
Pseudomonas aeruginosa ATCC 27853			-	-	-	-	-	-
Salmonella typhimurium ATCC 14028			-	-	-	-	-	-
Serratia marcescens ATCC 8100			-	-	-	-	-	-
Staphylococcus aureus ATCC 25923			-	-	-	-	-	-
Streptococcus pneumoniae		Clinical	-	-	-	-	-	-
Streptococcus pyogenes ATCC 19615			-	-	-	-	-	-
Vibrio cholerae		Clinical	-	-	-	-	-	-
Yersinia pseudotuberculosis		Clinical	-	-	-	-	-	-

The present invention is also used to detect viable virulent strains of *L. monocytogenes*. The PCR assay utilized in the present invention is effective in amplifying the above listed gene sequences from chromosomal DNA, which is not effective in distinguishing live *L. monocytogenes* from dead *L. monocytogenes*. However, amplification of the above listed gene sequences from mRNA by reverse transcription-PCR (RT-PCR) would only detect the presence of live, viable *L. monocytogenes*.

Because transcriptional regulators are essential components in the regulation of RNA synthesis and gene expression within bacteria, and because internalins play vital roles in listerial internalization, they may be potentially useful targets for treatment and control purposes. Therefore, it is also within the scope of this invention to use *L. monocytogenes* virulence-specific genes (*lmo0833*, *lmo1188*, *lmo0834*, *lmo1116*, *lmo2672*, *lmo1134*, *lmo0333*, *lmo2470*, and *lmo2821*) or their derivatives in the inhibition of growth, reduction of pathogenicity, treatment and prevention of listeriosis caused by virulent strains of *Listeria monocytogenes*.

For example, one possible treatment strategy would involve using pharmaceutically active agent(s) that would inactivate or alter the function of one or more of the proteins encoded by the above listed genes, which would either kill the virulent *L. monocytogenes* or render it susceptible to the host immune system. One possible vaccine strategy would involve altering one or more of the above listed genes or promoter(s) for one or more of the above listed genes such that expression of the encoded protein(s) would be completely disrupted or altered.

The said alteration or disruption of expression would render *L. monocytogenes* avirulent and effective as a live attenuated vaccine.

While the present invention has been described with reference to specific embodiments and exemplary bacteria species, it will be understood by those skilled in the art that a variety of changes may be made and the substitution of equivalents may be made without departing from the true spirit and scope of the present invention. Many modifications may be made to adapt a particular situation or a particular selected pathogen to the inclusive concept of the present invention. All such modifications or adaptations are intended to be within the scope of the claims appended hereto.

The complete disclosure of all references cited in this application are fully incorporated herein by reference.

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